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Stephen J. Elledge, Ph.D. 10/19/00

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## **Table of Contents**

<b>Page 1</b>	<b>Front Cover</b>
<b>Page 2</b>	<b>SF 298</b>
<b>Page 3</b>	<b>Foreword</b>
<b>Page 4</b>	<b>Table of Contents</b>
<b>Page 5</b>	<b>Introduction</b>
<b>Page 9</b>	<b>Body</b>
<b>Page 13</b>	<b>Conclusion</b>
<b>Page 16</b>	<b>References</b>
<b>Page 24</b>	<b>Appendix</b>
<b>Page 24</b>	<b>Table 1</b>
<b>Page 25</b>	<b>Table 2</b>
<b>Page 26</b>	<b>Table 3</b>
<b>Page 27</b>	<b>Table 4</b>
<b>Page 28</b>	<b>Personnel and Publications</b>

## Introduction

The eukaryotic cell cycle is regulated primarily at two points, in G1 prior to entry into S-phase and in G2 prior to entry into mitosis. The commitment to a round of cell division is made at a point in G1, referred to as the restriction point in mammalian cells<sup>(1-4)</sup> or START in yeast<sup>(5)</sup>. Passage through the restriction point depends critically on mitogen signals, but once this point is passed, cells are committed to S-phase and the remainder of the cycle in a mitogen independent manner<sup>(4)</sup>. Passage through the restriction point is thought to be the primary event controlling cell proliferation. Therefore, elucidating how positively and negatively acting genes function to regulate the G1/S transition and how mutations in these genes disrupt normal cell cycle control has been a primary focus of cancer research. Central to this focus has been the investigation of the role of cyclin-dependent kinases (Cdk) in the control of cell proliferation.

**Cyclins, Cdks, and positive growth control.** Cdks are protein kinases that require association with cyclins and phosphorylation for activity<sup>(5-8)</sup>. Cyclins promote cell cycle transitions via their ability to associate with and activate their cognate Cdks<sup>(5-12)</sup>. Cyclins D and E function in G1<sup>(6, 10, 13-16)</sup>, and overexpression of cyclin D1 or cyclin E shortens G1 and accelerates entry into S-phase<sup>(1, 3, 17, 18)</sup>. Amplification of cyclins, D1, D2 and E have been identified in several tumors<sup>(19-23)</sup>. Cyclin D1 was identified as the PRAD1 oncogene<sup>(24)</sup>. Cyclin A was identified as the site of integration of HBV in a hepatocellular carcinoma<sup>(25)</sup>. Taken together, these observations suggest that inappropriate activation of Cdks is a mechanism that cells frequently use to reach the oncogenic state.

Cyclins D1, D2, and D3 bind Cdk4 and Cdk6 kinases and can phosphorylate and inactivate Rb<sup>(6, 26-29)</sup>. Because D-type cyclins are required for proliferation only if cells have an intact Rb gene, it is thought that Rb inactivation is their primary role. Cyclin E binds to and activates Cdk2 and considerable evidence has accumulated indicating that cyclin E/Cdk2 is the primary kinase involved in the G1/S transition<sup>(14, 15, 30-33)</sup>. In addition, a close homolog of Cdk2 - Cdk3 - is also thought to play a unique role in the G1/S transition<sup>(31)</sup>. Cyclin A binds Cdk2 and Cdc2 and is required for both S-phase and the G2/M transition<sup>(34-36)</sup>, while cyclin B/Cdc2 complexes appear to be specific for control of mitotic entry.

Although Cdks are thought to be the critical regulators of cell proliferation, little is known about how cyclin/Cdk complexes regulate cell proliferation during development. In this regard, we have performed an analysis of the expression of the major cyclins during mouse embryonic development and in adult tissues. We have discovered that general cyclins E, A, B, and F are expressed in all proliferating tissues while the D cyclins are distributed in a pattern distinct for each cyclin but which is a subset of the general cyclins (Parker, Harper and Elledge, unpublished results). This is consistent with the notion that D cyclins are the primary initiators of cell cycle entry and orchestrate development. We have recently observed that cyclin D1 is the only D-type cyclin induced when breast cells proliferate during pregnancy. We and our collaborators in the Weinberg laboratory discovered that development of the breast during pregnancy is dependent upon cyclin D1<sup>(37)</sup>. As mentioned above, amplification of D-type cyclins is frequently observed in breast cancer. This provides a link between development and cancer and indicates that the developmental history of the breast is relevant to its susceptibility to tumorigenesis.

Since the controls utilized during development to regulate cell proliferation are similar to those utilized in maintenance of the non-proliferative state in differentiated tissues, it is likely that these controls are reactivated or overcome in cancer. Another example of this comes from our observation that cyclin D1 is expressed at extremely high levels in the retina and is required for its development<sup>(37)</sup>. Presumably the inability to properly develop the retina in cyclin D1 mutants reflects an inability to overcome Rb. In what is clearly more than a coincidence, the retina is the same tissue in which high frequency tumors arise in Rb mutant humans. It is therefore likely that the Rb protein is important in both development of that tissue and its maintenance in the non-proliferative state. Our understanding

of the links between development and cancer is in its infancy and is an area in which there is a great need to increase our knowledge base.

**Tumor suppressor proteins and negative growth control.** Rb and p53<sup>(29, 38)</sup> are the most well understood tumor suppressors. Mutations in these are found frequently in many human cancers<sup>(39,40)</sup>, and reintroduction of wild-type genes into p53<sup>-</sup> or Rb<sup>-</sup> tumor cells can suppress the neoplastic phenotype suggesting that loss of function of these genes contributes to tumorigenesis<sup>(28, 41, 42)</sup>.

Mutations in p53 are the most common lesions observed in human malignancies, occurring in greater than 50% of all tumors<sup>(39)</sup> including those of the breast. The percentage is much higher if loss of p53 function via association with viral oncoproteins (E1B of adenovirus and E6 of papilloma virus) or amplification of the p53 binding protein MDM2 are included<sup>(43)</sup>. p53 deficient mice are prone to the spontaneous development of a variety of tumor types<sup>(44)</sup>. Cellular responses to DNA damage such as apoptosis and the G1 checkpoint are dependent on p53<sup>(45-53)</sup>. p53 also controls a spindle checkpoint and prevents genetic alterations such as gene amplification<sup>(54, 55)</sup>. p53 regulates the expression of p21<sup>CIP1</sup>, an inhibitor of G1-cyclin/Cdks, in response to DNA damage<sup>(56-60)</sup>. Using a p21 knockout mouse, we have determined that p21 is required for full function of the G1 checkpoint in response to  $\gamma$ -irradiation, although there is residual checkpoint function<sup>(56)</sup>. Furthermore, these mice do not show the high rate of spontaneous tumor formation seen in p53-deficient mice. It is not clear whether p53's role in oncogenesis is through its checkpoint or apoptotic deficiencies, or a combination of these.

The current view of the role of Rb in the cell cycle is that hypo-phosphorylated Rb functions during G1 in part to block the activity of E2F and related transcription factors that are required for the expression of genes involved in S-phase<sup>(29)</sup>. Hyper-phosphorylation of Rb or association with DNA tumor virus oncoproteins such as E1A results in release of E2F and is correlated with passage into S-phase.

The above observations are consistent with a model in which increased cyclin/Cdk activity in tumors, whether by increased cyclin expression or decreased negative regulation, can overcome the cell cycle repression function of Rb via direct phosphorylation and inactivation of its growth inhibitory function. Rb therefore acts as a potential energy barrier in the pathway that cyclin/Cdks must overcome to activate cell cycle entry. Removal of the barrier (Rb) may reduce the levels of kinase activity required, but some Cdk kinase activity is still required for the process of DNA replication and can therefore act as a target of further negative regulation. In this model, p53 acts to reduce the frequency of mutations that lead to altered growth control and to kill cells that have undergone extensive damage or are inappropriately growing. To fully understand this aspect of cancer, cell cycle dysfunction, it is imperative that we have a complete understanding of the regulation of cyclin dependent kinases and their regulators in the tissues of interest.

**The cell cycle and development: potential roles for Cdk inhibitors.** Once proliferation and morphogenesis have constructed a particular structure, it is of paramount importance that the proliferative state cease and be replaced with a homeostatic state. While much attention has been focused on how cells enter the cell cycle, little is known concerning the strategies organisms employ to exit the cycle and maintain the non-proliferative state. This state is of great importance to an organism because the vast majority of its cells exist in a non-proliferative state throughout adult life. The inability to appropriately halt growth can lead to malformation during development, and to cancer. Thus, equally important in the execution of developmental programs is the arrest of growth once the program is complete. While the control of terminal differentiation promises to be complex, cell cycle arrest via inactivation of Cdks is likely to be a central feature. Recently a new class of Cdk regulatory molecules have emerged that are potential mediators of cell cycle exit and maintenance of the non-proliferative state. These are the inhibitors of cyclin-dependent kinases, CKIs. Currently two

structurally defined classes of CKIs exist in mammals that are exemplified by p21<sup>CIP1</sup> (57-60) and p16<sup>INK4/MTS1</sup> (61-65).

**Cyclin-dependent kinase inhibitors: mediators of negative cell cycle control.** Cdk inhibitory proteins are a group of proteins that associate with and inhibit Cdk. These versatile molecules have potential roles in cell cycle arrest, checkpoint function and development and are likely to cooperate with Rb, p53, and other negative regulators in maintaining the non-proliferative state throughout adult life. At the time of submission of this grant in December 1993, the first mammalian Cdk inhibitors p21<sup>CIP1/WAF1</sup> (57-60) and p16<sup>INK4a</sup> (61) had only recently been identified. Subsequently, we and others identified additional inhibitors including p27, p57, p15, p18, and p19 (refs 61-69). We identified p21<sup>CIP1</sup> in a two-hybrid screen designed to identify proteins that associate with Cdk2 (57). Importantly, this protein was simultaneously cloned by several other laboratories. p21 was cloned as a p53 activated gene by the Vogelstein laboratory (59), as a Cdk associated protein by the Beach laboratory (58), and as an S-phase inhibitory cDNA in senescent cells (60). Since then we and others have identified two other members of the p21 family, p27 and p57. p57, also known as KIP2 has been the focus of this study. It is expressed in the breast and is localized to 11p15.5 a locus involved in breast cancer (see below).

**Involvement of 11p15.5, the location of KIP2, in human cancers including cancer of the breast.** *Several chromosomal regions show frequent loss of heterozygosity (LOH) in breast tumors including but not exclusively 3p, 7q31, 11p15, 11q13 and 17p (reviewed in 96). The chromosomal location of KIP2, 11p15.5, marks it as a candidate tumor suppressor gene of the breast. The involvement of 11p15 in the breast is well documented (113-119). 35% of breast tumors show LOH at 11p15.5 (119) and this LOH is associated with poor prognosis (119). Furthermore, 11p15 LOH has been associated with metastasis (116) and there is evidence that 2 distinct breast tumor suppressor genes may reside at this locus (118). 11p15 has also been intensively investigated because of frequent LOH at this locus in a number of other human cancers including bladder, lung, ovarian, kidney, and testicular carcinomas (reviewed in 70). Several childhood tumors including Wilms' tumor, adrenocortical carcinoma, rhabdomyosarcoma, and hepatocellular carcinoma show specific loss of maternal 11p15 alleles, suggesting a role for genomic imprinting. Chromosome transfer experiments have also indicated a tumor suppressor gene resides at this locus, the WT2 gene involved in Wilms' tumor and possibly rhabdomyosarcoma (reviewed in 71), either of which could be due to loss of a Cdk inhibitor. In addition, rearrangements in the 11p15 region are found in Beckwith-Wiedemann Syndrome (BWS) which is characterized by numerous growth abnormalities, including macroglossia (enlarged tongue), gigantism, visceromegaly (enlarged organs) and an increased risk (7.5%) of childhood tumors (72,73). Genetic analysis indicates maternal carriers, also suggesting a role for genomic imprinting (reviewed in 74). Several features of KIP2 make it a reasonable candidate as a mediator of some phenotypes of BWS. First, a Cdk inhibitor could explain both overgrowth and tumorigenesis phenotypes. Furthermore, the expression pattern of KIP2 in mouse correlates with areas known to be affected in BWS including the tongue, kidney, muscle, and the eye. Third, KIP2 is imprinted and maternally expressed. Furthermore, LOH at 11p15 in Wilms' tumors are exclusively maternal, offering further support for the possibility that KIP2 might be the WT2 gene. LOH of the breast has not yet been examined for parental specificity of LOH. However, the potential for the existence of two tightly linked tumor suppressors (75) affecting the breast at 11p15 might complicate the analysis of parentally biased LOH depending on the relative frequency of the two events. Nevertheless, the biochemical properties of KIP2, its physical location and expression patterns suggest that it may be the tumor suppressor at 11p15.*

## A role for p57 in Beckwith-Wiedemann Syndrome.

As mentioned above, Beckwith-Wiedemann Syndrome (BWS) is a clinically variable disorder characterized by somatic overgrowth, macroglossia, abdominal wall defects and visceromegaly (83). Children with BWS are also susceptible to a variety of childhood tumors, including Wilms' tumor, hepatocellular carcinoma and rhabdomyosarcomas. The disease, which affects 1 out of every 13,700 live births each year, is genetically heterogeneous, with the majority of cases occurring sporadically. Familial cases, which represent ~15% of BWS patients, have helped establish a genetic linkage of BWS to human 11p15.5, where there is a large cluster of imprinted genes (84, 85). That defects in imprinted genes might explain the etiology of the syndrome was first suggested from observations that both familial and sporadic cases can be associated with 11p15.5 partial paternal uniparental disomies (UPDs) and trisomies with paternal duplications (86, 87).

Many of the key defects found in BWS patients could be explained by alterations in the control of cell proliferation, either in the context of organogenesis or tumorigenesis. Thus, candidate genes should be tied in some respect to the control of cell proliferation as well as being imprinted. Two candidate imprinted genes with these properties map to 11p15.5, *IGF2* and *p57<sup>KIP2</sup>*. These genes have strikingly similar patterns of expression during development in mice and are expressed in all of the tissues affected by BWS (88, 89, 90).

*IGF2* encodes a fetal-specific growth factor that is paternally expressed in both mice and humans (91, 92, 93). When the expression of *IGF2* was examined in BWS patients, it was shown to be elevated as the result of deregulation of its imprinting in some, but not all cases with normal karyotypes (94, 95). Furthermore, paternal trisomies and UPDs would be expected to double the expression of *IGF2* and could potentially account for overgrowth observed in these cases of BWS.

Maternally inherited loss-of-function mutations in the *p57<sup>KIP2</sup>* gene have also been identified in ~5-15% BWS cases examined (96, 97) and 30%-50% of familial cases (98). *p57<sup>KIP2</sup>* encodes a member of the CIP/KIP family of cyclin-dependent kinase inhibitors (CKIs), and is maternally expressed in all mammals examined to date (99, 89, 90, 100, 101). CKIs of this class inhibit G1/S phase cyclins, and the absence of *p57<sup>KIP2</sup>* has been shown to affect the ability of cells to exit from the cell cycle. The gene lies ~800 kb from *IGF2* in both mouse and human (102, 103, 104).

A small number of patients (<1%) have been identified with balanced translocations or inversions 3' of *p57<sup>KIP2</sup>* in a neighboring imprinted gene, *KvLQT1* (105, 106, 107). This gene encodes a voltage-gated potassium channel that is maternally expressed in humans in all tissues except the heart, but mutations in the gene have been implicated only in the cardiac arrhythmia long QT syndrome, not BWS (108, 102). It has been suggested that the regulation of other genes in the locus, particularly *p57<sup>KIP2</sup>* and *IGF2*, may be disrupted by the translocations. Indeed, in one such family, *IGF2* expression has been shown to be biallelic (109). Finally, the methylation imprint and expression of a recently described paternally expressed transcript within an intron of *KvLQT1*, *LIT1*, has been shown to be disrupted in over 50% of BWS patients examined (110, 111, 112). These groups suggest that this transcript may mediate the imprinting of other genes in the locus, although they disagree on the likeliest candidates (110, 111, 112). Several mouse models that shed light on the etiology of BWS have been generated. These studies have confirmed that embryonic growth in mice is very sensitive to the levels of the growth factor IGFII. When a mutated copy of the *Igf2* gene was inherited paternally, the offspring were 60% the size of their wild-type littermates (91). In contrast, in mice in which the cis-acting sequences that control *Igf2* imprinting were deleted (*H19\_13*), *Igf2* was expressed from both parental chromosomes, and the offspring displayed somatic overgrowth and placentomegaly, but none of the other symptoms of BWS (113, 114). This mutation raised the tissue levels of IGFII 2-fold, but had a less pronounced effect on its circulating levels.

Mutations in the type 2 *Igf2* receptor gene (*Igf2r*), whose product binds IGFII and targets it for lysosomal degradation also caused elevation of IGFII (115, 116, 117). Circulating IGFII levels were elevated ~4-fold, and embryos died late in gestation with many, but not all, of the phenotypes of BWS, such as somatic overgrowth, placentomegaly, heart hypertrophy, omphalocele and adrenal cysts. In *Igf2r* and *H19\_13* double mutants (114), IGFII levels were increased 7-11 fold, and the severity of



the phenotypes were more pronounced than in either single mutant. In these mice, however, the macroglossia, renal dysplasia and adrenal cytomegaly commonly found in BWS patients were missing. Overexpression of IGFII has also been achieved in mice carrying *Igf2* transgenes (118). These animals exhibited overgrowth, polydactyly and polyhydramnios, all symptoms of BWS patients. Together, these animal models support the hypothesis that BWS results from elevated expression of *IGF2*.

The impact of loss-of-function mutations in *p57<sup>Kip2</sup>* has also been examined in mice [(119, 120). These mice show abdominal wall defects reminiscent of those seen in *Igf2r/H19* double mutants. They also display a unique set of defects such as renal dysplasia and adrenal cytomegaly, defects seen in BWS patients but not observed in mice with elevated IGFII expression. The somatic overgrowth commonly associated with BWS, however, was not observed. In addition, phenotypes not previously associated with BWS, such as lens and gastrointestinal tract abnormalities as well as skeletal defects were present in *p57<sup>Kip2</sup>*-null mice.

These mouse models have failed to provide a good explanation for the fact that BWS patients with loss of imprinting of *IGF2* and those with mutations in *p57<sup>KIP2</sup>* are phenotypically indistinguishable. Zhang et al. (120) suggested that IGFII and p57 may act in opposing manners to control cell proliferation during development of human fetuses; that is, that a gain of function of *IGF2* may act similarly to a loss of function of *p57<sup>KIP2</sup>*. We reasoned that if IGFII and p57 act antagonistically during development, a double mutant in which both BWS-potentiating mutations are present might exhibit phenotypes that are more severe than the sum of those in the single mutants. Such a mouse strain would mimic patients with UPD of 11p15.5, with respect to *IGF2* and *p57<sup>KIP2</sup>*. To generate such a mouse we bred a loss-of-function *p57<sup>Kip2</sup>* mutation to an *Igf2* loss-of-imprinting mutation (*H19\_13*) and screened for meiotic recombination between these tightly linked genes in the next generation. The double mutant mice exhibit aspects of BWS that have not been observed in other mouse models, such as macroglossia. In addition, they show an exacerbation of the placental and kidney dysplasias caused by the *p57<sup>Kip2</sup>* mutation alone. Significantly, we observed that a null mutation in *Igf2* can overcompensate for these severe placental and kidney dysplasias, leading us to suggest that the two genes act in an antagonistic manner in some tissues in the mouse.

The goals of our work were: 1) to determine whether p57 is imprinted in the breast, 2) to construct mice lacking p57, 3) to analyze the phenotype of mice lacking p57, 4) to analyze the role of the QT domain in p57 function by looking for binding proteins, and 5) to characterize the regulation of p57 and 6) to look for additional CKIs in the breast. To date, we have made significant progress on these initial goals. Our progress in these areas is summarized below.

## Body

### **Aim 1: Determination of p57 imprinting status in the breast.**

We have completed this Aim and described this in last years report..

**Aim 2: Construction of mice deficient in KIP2.** We completed this aim and discussed it in last years report.

### **Aim 3: Analysis of p57 mutants animals.**

We have completed an exhaustive analysis of the mutant phenotypes present in the p57 mutant and p57/27 double and p57/p21 mutant animals in last two years and discovered multiple roles for these proteins in eye, lung, and muscle development. In the past year we have investigated the possible overlap between p57 loss and increases IGF2 growth factor expression in mouse models for the cancer syndrome BWS by making mice mutant for both p57 and H19, a negative regulator of IGF2 imprinting. H19 mutations express 2-fold more IGF2 and IGF2 overexpression has been implicated in BWS. The phenotypes of mice lacking these two genes are discussed below in the order in which we detected them.

### A. Generating a $p57^{Kip2}/H19$ double mutant

The phenotypic consequences of mutations in  $p57^{Kip2}$  and  $H19$  are manifested only when the genes are inherited from mothers. Because these genes lie ~900 kb apart in the mouse, a double mutant strain could be generated by meiotic recombination between the existing mutations. Towards that end, we crossed heterozygous  $p57^{Kip2+/-}$ ;  $H19\Delta13^{-/+}$  males to C57BL/6 females and screened progeny for animals that were heterozygous for both  $p57^{Kip2}$  and  $H19\Delta13$  ( $p57H19$ ). Among 481 offspring, we identified two  $p57H19$  recombinants, a frequency consistent with the estimate of the genetic distance between the genes (0.5 cM). One recombinant, a male, was fully viable and fertile and was used to establish the line. The other recombinant, a female, failed to give birth to viable  $p57H19$  mice.

Loss of *Igf2* imprinting, and the absence of maternal  $p57^{Kip2}$  and  $H19$  expression were confirmed by RNase protection and RT-PCR assays. The imprinting and expression of *Kvlqt1* was unchanged (data not shown).

### B. Perinatal Lethality

Maternal inheritance of a null allele of  $p57^{Kip2}$  is lethal, with 10% of offspring dying *in utero* and the remainder dying within the first two weeks after birth (120). We were unable to recover live  $p57H19$  mice at birth, suggesting that the double mutant phenotype is more severe. Furthermore parturition invariably occurred at 19 d.p.c., at least one day earlier than in  $p57^{Kip2}$  single mutants. At 18.5 d.p.c.,  $p57H19$  embryos were present at approximately the expected frequency (Table 1), suggesting that death occurred during or shortly after delivery.

### C. Prenatal Growth

One of the characteristics of BWS is prenatal somatic overgrowth. It had been shown previously that offspring inheriting the  $H19_{13}$  mutation maternally are born 30% bigger than their wild-type littermates (113, 114).  $p57^{Kip2}$  mutants, on the other hand, display no somatic overgrowth at birth (119, 120). Although  $p57H19$  embryos were indistinguishable in weight from wild-type littermates at e18.5, they were ~20% larger at e16.5-17.5 just as was seen in the single  $H19\Delta13$  deletion at the same time (113). It is conceivable that the gain in growth rate mediated by the loss of *Igf2* imprinting is compromised later in gestation by  $p57^{Kip2}$ -induced defects.

BWS patients often display specific organomegaly, most often affecting the tongue and adrenal glands, and less frequently the liver, kidney and heart. To distinguish specific organ overgrowth from generalized overgrowth in  $p57H19$  mutant mice, we calculated organ weights at 18.5 d.p.c. as a percentage of total body mass. By this criterion, neither  $H19_{13}$  nor  $p57^{Kip2}$  mutant mice showed specific organ overgrowth (data not shown). In  $p57H19$  offspring the only organ that displayed significant overgrowth was the tongue which was 122% of wildtype ( $p<0.001$ ) (Table 2). If we tabulate those animals whose normalized growth was one standard deviation above the mean of their wild-type littermates, overgrowth of the kidney, heart, as well as overall somatic overgrowth, are observed more frequently in  $p57H19$  mutants than in wild-type littermates (Table 3).

### D. Placental defects

The most dramatic overgrowth phenotype observed in  $p57H19$  embryos was placentomegaly, with 18.5 d.p.c. placentas weighing on average 190% of those of wild-type littermates. On histological analysis, mutant placentas were highly disorganized in the labyrinthine layer, where both  $p57^{Kip2}$  and *Igf2* are expressed. Mutant placentas also displayed fibrin cysts, apoptotic cells and large accumulations of red blood cells. A similar disorganization was seen at 16.5 and 17.5 d.p.c., suggesting that this effect occurs before the degeneration of the organ that normally occurs in late gestation. It is unclear whether red blood cells accumulate because of the tubule networks through which maternal and fetal blood flow are not established, are breaking down through cell death, or are blocked by surrounding cellular overgrowth.  $p57^{Kip2}$  single mutant animals showed related morphological placental defects, including reduced vascularization of the labyrinthine zone caused by

an overproliferation of trophoblast cells. In addition, the occurrence of hyaline membranes, a response to endothelium damage, was observed and was thought to result in a blockage of the blood supply [(121). The *p57H19* phenotype that we observed, however, was far more severe.

In *p57H19* mutants there was a direct correlation between the placental weights and disruptions in placental cellular architecture, suggesting that the observed placentomegaly is caused primarily by the increase in red blood cell volume. Interestingly, there was actually a modest positive correlation ( $r=0.37$ ) between the disorganization of the placenta, as reflected by its weight, and the size of the embryo. Thus the placental dysmorphologies did not compromise fetal growth.

The placental morphology of BWS patients has been examined in a few cases where disease was anticipated prenatally or following stillbirths or neonatal death. In these cases, placentas contained large cysts in stem villi. Some of the terminal villi in individual cases were filled with blood, and the trophoblast layer was hyperplastic in some, but not all, cases (122). Despite the significantly different architectures of the human and mouse placentas, these phenotypes are similar to those we observe in the *p57H19* mice.

### E. Kidney Dysplasia

Kidneys in *p57H19* mice were underdeveloped in the medullary region, where *p57<sup>Kip2</sup>* is normally expressed. This region normally consists of stromal mesenchymal cells surrounding a duct collection system. In the mutant mice, fewer collection ducts and dilated renal pelvises were observed. Kidneys varied from normal to kidneys in which there was no evident medulla or which contained large cystic-looking regions disrupting the medulla. In general, the degree of disorganization in the medulla was significantly more pronounced than had been observed in the *p57<sup>Kip2</sup>* single mutant strain, where slightly fewer collection ducts, less mesenchymal tissue and a reduced medulla as well as more stromal cells were reported (120).

There was a strong correlation between the severity of the placental and kidney phenotypes within individual *p57H19* mutant embryos. It is possible that the disorganized placenta, through ineffective nutrient transfer, results in the defects in kidney development. Alternatively, both tissues might independently be sensitized to excess IGFII in the absence of *p57<sup>Kip2</sup>* expression. The variability in the kidney phenotype was observed between animals, not between the two kidneys of a single animal, suggesting that genetic background could play a critical role in its severity.

Renal dysplasia is a common feature of BWS. Specifically, patients with a reduction in the collecting ducts and numerous fluid filled cysts as well as hypertrophy of the mesenchymal tissue have been reported. In humans, renal dysplasia is thought to result from abnormal metanephric differentiation (123). We did not observe hypertrophy in *p57H19* kidneys, which may reflect the fact that most BWS kidneys analyzed are from adults, not fetuses. In all other respects, *p57H19* dysplastic kidneys appear to mimic most of the aspects of the human disease. In contrast to the kidney dysplasias, we saw no defects in adrenal architecture or size in *p57H19* mice. This was surprising in light of the previous finding of adrenomegaly in *p57<sup>Kip2</sup>* mice (120).

### F. Abdominal Wall Defects

Closure of the ventral abdominal wall in mice occurs around 16.5 d.p.c. and is preceded by the retraction of the midgut into the abdominal cavity. Failure of the intestine to retract results in an umbilical hernia whereas failure of the abdominal wall to close results in an omphalocele, in which the midgut protrudes from the abdomen. Around 60% of BWS patients need surgical correction of omphalocele at birth, and another 32% display umbilical hernias (Table 3). One or the other of these phenotypes was seen in 55% of *p57H19* embryos, consistent with the previous report that *p57<sup>Kip2</sup>* mutants display omphalocele as well as body wall muscle dysplasia. Although this condition is not observed in *H19 $\Delta$ 13* mutants, it is seen in *Igf2r* and *Igf2r/H19 $\Delta$ 13* double mutants, where IGFII levels are elevated >2-fold (115,116,117,114).

### G. Cleft Palate and Skeletal Abnormalities

BWS patients occasionally display cleft palate, a condition that results from the failure of the palatal shelves to elevate, rotate or fuse. Previous studies had detected cleft palate in *Igf2r/H19Δ13* as well as in *p57<sup>Kip2</sup>* mutant mice but not in either *H19Δ13* or *Igf2r* mutant mice, implying that either significantly elevated IGFII or reduced p57 could lead to the developmental defect. In *p57H19* mutants, the frequency and severity of cleft palate, which was detected in 26% of offspring, was similar to that seen in *p57<sup>Kip2</sup>* single mutants (Table 3), suggesting that the primary cause of cleft palate is loss of *p57<sup>Kip2</sup>* function. It has been proposed that the cleft palate in *p57<sup>Kip2</sup>* mutants results from the failure of *p57<sup>Kip2</sup>* mutant cells to exit the cell cycle, and to undergo apoptosis (119).

Polydactyly has been observed in ~5% of BWS patients (Table 3). This phenotype has also been observed in mice displaying elevated IGFII, with the frequency and severity increasing with the dosage of IGFII. *p57<sup>Kip2</sup>* single mutants do not display polydactyly whereas *H19Δ13* mice do display postaxial polydactyly 68% of the time on a 129/Sv background (114). As expected for an *Igf2*-dependent phenotype, *p57H19* double mutants also show postaxial polydactyly in two genetic backgrounds examined (Table 4).

#### H. The Role of *Igf2* in the *p57H19* phenotype

The dramatic increase in the severity of kidney and placental dysplasia in *p57H19* double mutants occurred in animals over-expressing IGFII. Although the somatic overgrowth observed in *H19Δ13* mutant animals was genetically shown to be the consequence of the over-production of IGFII (113), it is possible that the novel defects observed in *p57H19* mice are due to effects of the *H19* deletion on genes other than *Igf2* or possibly even due to the loss of the *H19* mRNA itself. If the new phenotypes are due to IGFII overproduction, a reduction in IGFII levels should ameliorate them. To test this, *p57H19* females were crossed to *Igf2<sup>+/-</sup>* males with a null allele of *Igf2* (*p57H19; Igf2*) (91). Previous studies had determined that while the expression of *Igf2* mRNA from the maternal chromosome in *H19Δ13* mice in mesodermal tissues such as skeletal muscle and heart was essentially equivalent to that on the paternal chromosome, its derepression was less pronounced in endodermal tissues such as liver (113). Furthermore, the circulating levels of IGFII were only modestly elevated (114). Thus overall maternal expression of IGFII in *p57H19; Igf2* mutants is lower than that from a wild-type paternal chromosome.

At 18.5 d.p.c. triple mutant fetuses (*p57H19; Igf2*) were indistinguishable in size from wild-type, or *p57H19* fetuses (Table 2). In contrast, *Igf2<sup>+/-</sup>* offspring were 58% the size of wild-type, as had been observed previously (91). Thus in the absence of *p57<sup>Kip2</sup>*, prenatal growth was relatively insensitive to the ~2-fold differences in the levels of IGFII between *p57H19* and *p57H19; Igf2* mice. The complete absence of IGFII in *p57<sup>Kip2</sup> -/+; Igf2<sup>+/-</sup>* double mutants, on the other hand, results in embryos that are the same size as their *Igf2<sup>+/-</sup>* litter-mates (data not shown).

In contrast to the insensitivity of somatic growth to the reduction in *Igf2*, the placental overgrowth and dysplasia in *p57H19* embryos were almost completely suppressed in triple mutants (Table 2). That is, the mean weight of placentas did not differ from that of wild-type littermate placentas, but did differ substantially from that of the *p57H19* placentas, which were 53% over-sized in this cross. On histological examination, triple mutant placentas were morphologically normal, lacking even the moderate level of disorganization seen in *p57<sup>Kip2</sup>* single mutants. Thus the placental dysplasia, which is *p57<sup>Kip2</sup>*-dependent, can be suppressed by reducing the levels of IGFII.

The same situation appears to hold for the kidney dysplasia. None of the triple mutants had cystic kidneys, and the only discernible differences observed in a few fetuses were a slightly less developed medulla and a slight increase in mesenchymal tissue between the renal tubules. However, in most animals, the kidneys were less affected than in *p57KIP2* animals. Finally, the macroglossia observed in the *p57H19* mutant was completely absent in the triple mutant, indicating that overgrowth of the tongue is also *Igf2* dependent (Table 2). This is consistent with the finding of macroglossia in *Igf2* overexpressing transgenic mice (118).

The frequency of abdominal wall defects in *p57H19* mice was unaffected by reducing the levels of *Igf2* expression in *p57H19; Igf2* mice (Table 4). This result is somewhat surprising, as omphalocele was induced in the presence of highly elevated levels of IGFII in *Igf2r* and *Igf2r/H19* mutant mice. Thus, in *p57H19* mice, this defect appears to be entirely attributable to *p57<sup>Kip2</sup>* loss-of-function. Likewise the frequency of cleft palate is not affected by a loss of *Igf2* in the triple mutant, even though cleft palate was observed in *Igf2r/H19* double mutants. Lastly the postaxial polydactyly that is observed in both *p57H19* and *H19* mutants was completely rescued by the reduction in *Igf2* expression.

#### **Aim 4. Analysis of the QT domain.**

We screened for proteins that bind to C-terminus of *p57* by the two hybrid system. We did not find any specific interactors in this screen and have discontinued them.

#### **Aim 5. Transcriptional control of *p57<sup>KIP2</sup>*.**

We completed this aim and discussed it in last years report.

**Aim 6. Identification of new CKIs and other potential regulators of Cdks from normal breast.** This aim proposes to look for additional Cdk binding proteins in the breast using the two hybrid system and breast cDNA libraries. Our screens did not turn up any new proteins that were not already identified in previously published screens using libraries from other tissue sources.

### **Conclusion:**

The last year was a very productive one for our lab and the cell cycle field in general. Our work funded under this grant allowed us to establish the role of *p57<sup>KIP2</sup>* in mouse development and the human cancer and overgrowth syndrome BWS. *p57<sup>KIP2</sup>* clearly acts as a regulator of cell proliferation in the adrenal gland, the lens epithelia, and certain chondrocytes. The partial dependency on *p57<sup>KIP2</sup>* for reducing cell proliferation reveals the redundant mechanisms used to limit tissue growth. A similar situation is observed in cell culture where agents that induce cell cycle arrest immediately increase levels of certain CKIs and subsequently reduce the levels of the cyclins and Cdks. While undergoing the process of reducing Cdk activity during differentiation, the absence of CKIs may allow additional cell cycles to occur before Cdk activity is sufficiently reduced to block cell cycle entry. In addition, other CKIs may provide Cdk inhibitory functions in the absence of *p57<sup>KIP2</sup>*, as we have shown here in the lens development of *p27/p57* double mutant mice.

*CKIs* are the ultimate effectors of signal transduction pathway intended to bring about cell cycle arrest and the patterns of expression during embryonic development suggest that particular *CKIs* play important roles in terminal differentiation in a tissue specific manner. However, the fact that mice lacking single *CKIs* display surprisingly few developmental phenotypes has brought into question the essential nature of *CKIs* for cell cycle arrest and differentiation. Our studies **funded by this grant demonstrate** that two *CKIs*, *p57* and *p21* cooperate to control proliferation and differentiation in multiple tissues and reiterates the critical importance of *CKIs* to cell cycle control during development. The use of multiple *CKIs*, each controlled through distinct signaling pathways, provides a flexible mechanism to control proliferation in a cell type specific manner. It is likely that the combinatorial use of *CKIs* will emerge as one of the principal means through which cell cycle arrest and differentiation are integrated during development.

The more recent studies on the roles of IGF2 and *p57* was prompted by the clinical findings in BWS that mutations in *p57<sup>KIP2</sup>* and over-expression of *IGF2* have each been proposed as causes of the disease. Yet the phenotypes of the mouse models for loss of function of *p57<sup>KIP2</sup>* and loss of

imprinting of *Igf2* are readily distinguished (113, 119, 120, 124) McLaughlin et al (124) have shown that mice containing a UPD of a large portion of the distal end of mouse chromosome 7 die at 9.5 d.p.c., presumably as a consequence of the reduced expression of *Mash2*, a gene that is required for placental development and is maternally expressed (125, 126). By creating a mouse model in which only the two candidate genes are affected, we hoped to gain more precise insight into the ways in which mis-regulation of these two apparently unrelated genes could lead to the same disease.

The *p57H19* mice exhibited a dramatic increase in the severity of several BWS phenotypes such as placental overgrowth and dysplasia and kidney defects. Furthermore, macroglossia, one of the hallmarks of BWS, was seen in these mice. A direct interaction between the *p57<sup>Kip2</sup>* and *Igf2* pathways is implied by the ability of the *Igf2* mutation to compensate for the placental and kidney dysplasias that arise from mutations in *p57<sup>Kip2</sup>*. Thus in the absence of *p57<sup>Kip2</sup>*, the disorganized development of those tissues is enhanced in an *Igf2*-dependent manner. When signaling through *Igf2* is reduced, the loss of *p57<sup>Kip2</sup>* is no longer detrimental. Thus our analysis of *p57H19* mice suggests a resolution to the dilemma of how both loss-of-function mutations in *p57<sup>KIP2</sup>* and gain-of function mutations in *IGF2* can lead to BWS. That is, in the mouse the two genes act in an antagonistic manner in a subset of the tissues in which they are co-expressed.

A pathway that is affected by both genes is the one that regulates G1 cell cycle progression. Both IGFII and p57 are involved in regulating the progression of cells through the G1/S phase of the cell cycle, with IGFII promoting the G1/S transition (127) and p57 inhibiting the G1 cyclin-dependent kinases (CDKs) (89, 90). p57 has not been implicated in regulating embryo size, but is involved in the cell cycle arrest that precedes terminal differentiation of tissues such as skeletal muscle, lens and placenta (120, 121, 128). IGFII, on the other hand, is a direct regulator of fetal growth, and has been shown to promote progression through the G1 phase of the cell cycle, possibly through its ability to increase the level of the G1 cyclin D1 (127). The decision to proceed through the G1 to S phase transition checkpoint is controlled by the ratio of cyclin/Cdk complexes to CKIs, which determines the overall activity of G1-cyclin/Cdk complexes. It is possible that the exacerbation of the *p57<sup>Kip2</sup>* phenotype in the presence of excess IGFII and its alleviation when the concentration of the growth factor is reduced reflects cell-type specific sensitivity to Cdk activity in placenta and kidney. Excess IGFII in the absence of *p57<sup>Kip2</sup>* could lead to hyperproliferation, as seen in the placenta, to increased apoptosis, which is observed in both *p57<sup>Kip2</sup>* and *p57H19* mutant placentas and kidneys, or to a failure to differentiate, as is suggested by the reduction of medullary cells in the kidney. Increased apoptosis has also been reported in the palatal shelves of *p57<sup>Kip2</sup>* single mutants, and presumably results from alterations in the orderly progression through cell cycle checkpoints (119).

Several *p57<sup>Kip2</sup>*-dependent phenotypes such as cleft palate and omphalocele are neither enhanced by over-expression of *Igf2*, nor rescued by its absence. It may be that the insensitivity of cleft palate and omphalocele phenotypes to changes in IGFII levels reflects the fact that these tissues regulate the CDK to CKI levels using growth factors other than IGFII. On the other hand both cleft palate and omphalocele are observed in the most severe IGFII gain-of-function mouse model, the *Igf2r/H19* double mutant, where IGFII levels were 7-11 fold higher than normal (114). These animals also displayed the lens abnormalities and skeletal defects that were seen in the *p57<sup>Kip2</sup>* mutants but are not associated with BWS. Thus it may be that in the presence of *p57<sup>Kip2</sup>*, very high levels of IGFII are required to alter the CDK to CKI balance.

One of the surprising findings in this study was that the somatic overgrowth that has been shown to be *Igf2*-dependent in *H19Δ13* mice was less pronounced in *p57H19* mice. We considered the possibility that the lack of somatic overgrowth was due to the failure of the dysplastic placenta to provide nutrients to the embryo in the later stages of gestation. However the subset of *p57H19* mice in which the placenta was relatively normal were not oversized at birth (data not shown). Furthermore there was a modest positive correlation between the degree of placental hyperplasia and the size of the embryos in general ( $r=0.37$ ). The fact that *p57<sup>Kip2</sup>* mutants are reduced in size in the presence of a null mutation in *Igf2* argues that the *p57<sup>Kip2</sup>* mutation does not completely desensitize the embryo to

changes in IGFII concentration. Rather it may be that the ~2-fold changes in IGFII expression between *p57<sup>Kip2</sup>*, *p57H19* and *p57H19; Igf2* mice do not shift the ratios of CDKs to CKIs sufficiently to effect a change in overall growth rate. Of the Cip/Kip family of CKIs, only *p27<sup>Kip1</sup>* has been directly implicated in overall fetal growth (129, 130, 131, 132).

Our results suggest a model in which p57 and IGFII act antagonistically in the control of cell proliferation and development in several tissues affected in BWS patients including the tongue, kidney, and placenta. The affected tissues in the mouse are those in which some rate-limiting step is controlled by the two growth regulators, while other tissues, like the liver, presumably utilize other positive or negative growth signals. Although the nature of these other pathways are unknown, IGFI has been shown to function redundantly with IGFII to promote cell growth (133). Likewise several instances of redundancy between Cdk inhibitors have been observed. For example, p21 and p57 are redundant for control of muscle and lung development (128); p27 and p57 are redundant for control of lens development (121); and p18 and p27 work together to control pituitary gland, spleen, and thymus embryonic growth (134). We propose that BWS phenotypes are observed when the overall balance of regulators are shifted in favor of proliferation by either an increase in IGFII, or a decrease in p57. While a shift in the balance of other regulators could potentially cause a BWS-like phenotype, it is likely that the differing tissue specificity of the other regulators would prevent them from producing a phenotype recognized as BWS.

The fact that the *p57H9* double mutant does not completely recapitulate the BWS phenotype probably reflects species-specific differences in the tissues in which the two genes act directly in opposition to one another. Furthermore because the changes in the cell number in a given organ due to a change in the rate of proliferation is an exponential function, the more rounds of cell division a tissue undergoes, the greater will be the effect of a small increase in proliferation rate. Since human organs such as the tongue, kidney and placenta undergo significantly more cell division, a relatively small change in proliferation rates afforded by p57 loss or increased IGFII may have a more pronounced effect in humans relative to the mouse.

The variability in the phenotypes in the *p57H19* mice is reminiscent of the highly variable phenotypes of BWS patients (Table 3). The mice in this study were not on completely inbred backgrounds, and at least some of the variability we observed could be attributed to genetic modifiers. Another explanation for the variability in the human syndrome is suggested by the curious clinical finding that 10 sets of female identical twins have been reported who are discordant for BWS (135). Although the high level of discordancy is not understood, it has been suggested that disruptions in epigenetic mechanisms such as X-inactivation and imprinting might explain the occurrence, an explanation that could extend to sporadic cases as well.

In conclusion the analysis of the defects in *p57H19* mice demonstrates that some but not all tissues are highly sensitive to the ratio of p57 and IGFII. Perturbations in the levels of either protein may be sufficient to generate the variable range of phenotypes in BWS.

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## Appendix

### Tables 1-4

Table 1. Genetic crosses

Cross	# litters	Genotype	#embryos
<u><i>p57H19<sup>+/-</sup></i> X C57BL/6</u>	24		170
		<i>p57H19</i>	99
		+/+	71
<u>C57BL/6 X <i>p57H19<sup>+/-</sup></i></u>	6		52
		<i>p57H19</i>	12
		+/+	14
<u><i>p57H19<sup>+/-</sup></i> X <i>Igf2<sup>+/-</sup></i></u>	10		75
		<i>p57H19</i>	14
		<i>p57H19; Igf2<sup>+/-</sup></i>	26
		<i>Igf2<sup>+/-</sup></i>	15
		+/+	20
<u><i>H19Δ13<sup>+/-</sup></i> X C57BL/6</u>	4		26
		<i>H19Δ13<sup>+/-</sup></i>	12
		+/+	14
<u><i>p57<sup>Kip2+/-</sup></i> X C57BL/6</u>	4		30
		<i>p57<sup>Kip2+/-</sup></i>	16
		+/+	14
<u><i>p57<sup>Kip2+/-</sup></i> X <i>Igf2<sup>+/-</sup></i></u>	2		22
		<i>p57<sup>Kip2+/-</sup></i>	2
		<i>Igf2<sup>+/-</sup></i>	7
		<i>p57<sup>Kip2+/-</sup>; Igf2<sup>+/-</sup></i>	6
		+/+	7

Embryos of the crosses indicated were genotyped between 12-18.5 d.p.c. Female parent indicated first.



**Table 2**      *Growth of p57H19 mutant embryos*

Tissue	P57H19 (BL/6) n = 20	p57H19 (BL/6-129) n = 8	Igf2 <sup>+/-</sup> (BL/6-129) n = 10	p57H19; Igf1 <sup>+/-</sup> (BL/6-129) n = 19
Embryo	1.04	1.06	0.58*	0.93
Placenta	1.94*	1.53*	0.55*	1.0
Tongue	1.22*	1.11	0.86	1.00
Heart	1.03	1.17	1.03	1.16
Kidney	1.13	N.D.	N.D.	N.D.
Liver	0.83	0.98	0.75	0.89

The wet weights of embryos and internal organs of the genotypes indicated are expressed as a fraction of that of wild type littermates. The p57H19(BL/6) animals were derived from crosses to C57BL/6 males; all others were derived from a p57H19<sup>+/-</sup> x Igf<sup>+/-</sup> cross. (N.D.). Not determined. \*P < 0.05.

**Table 3**      *Summary of phenotypes in BWS animal models*

Phenotypes	Percent Observance		
	BWS	p57H19	+/+
Macroglossia	95	60(12/20)	11(1/9)
Adrenal defects	94	0	0
"      cysts	69	0	0
Placentomegaly	92	96(27/28)	13(2/15)
Visceromegaly	85	16(3/19)	11(1/9)
"      nephromegaly	69	40(8/20)	13(1/8)
"      cardiomegaly	N.D.	15(3/20)	13(1/8)
Somatic overgrowth	60	39.2(11/28)	13(2/15)
Renal dysplasia	59	61.5(8/13)	0
Abdominal wall defects	60	4(1/23)	0
"      "      umbilical hernia	32	48(11/23)	0
Cleft palate	7.1	26(6/23)	0
Subcutaneous cleft palate	20	N.D.	N.D.
Cardiac defects	20	39(5/13)	0
Polydactyly	5.5	30(7/23)	0

The frequency of the phenotypes is expressed as a percentage of all individuals examined. The BSW data are adapted from Eggenschwiler et al. (1997). The p57H19 and +/+ data are taken from 18 d.p.c. p57H19 x C57BL/6 litters. (N.D.) Not determined.

**Table 4.**      *Effect of reducing IGFII in p57H19 mice*

Phenotype	Genotype	
	p57H19	p57H19; Igf2
Postaxial polydactyl	50(4/8)	0(0/15)
Cleft palate	12.5(1/8)	20(3/15)
Omphalocele	37.5 (3/8)	20(3/15)
Umbilical hernia	25(2/8)	27(4/15)

The frequency of the phenotypes in 18 d.p.c. fetuses are expressed as a percentage of all individuals examined.

## Personnel

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